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Short Communication

Detection and identification of *Leishmania* isolates from patients with Cutaneous Leishmaniasis (CL) in Isfahan (central region of Iran) by PCR method

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ABSTRACT

Leishmaniasis is caused by parasitic protozoa of the genus *Leishmania*. Cutaneous leishmaniasis (CL) is a complex disease with wide spectrum of clinical manifestations. In order to identify leishmania species causing CL in Isfahan by a definite molecular technique (PCR method), this study was undertaken over 2010- 2011. 124 Patients with suspicious lesion of Leishmaniasis and positive direct smear from lesion were selected. Samples were cultured in NNN and RPMI 1640 media Negative and positive control and clinical samples was applied for PCR in the same condition. In the next step, standard PCR was carried out using classic protocol. From 124 patients, 111 (89.51%) cases were infected as *L. major* and 12 (9.67%) cases were infected by *L. tropica*, However only in one patient simultaneous infectious with both *L. major* and *L. tropica* was identified by PCR techniques which could not be possible in microscopy. *L.major* was the most prevalent species in the studied patients (p-value<0.001).

Keywords: Characterization, Cutaneous Leishmania, *L. major, L. tropica, PCR*

INTRODUCTION

Leishmaniasis is caused by parasitic protozoa of the genus *Leishmania*. Humans are infected via the bite of phlebotomine sandflies, which breed in forest areas, caves, or the burrows of small rodents. Cutaneous leishmaniasis (CL) is a complex disease with wide spectrum of clinical manifestations. More than 90 percent of CL cases live in the following countries: Afghanistan, Saudi-Arabia, Aljazeera, Brazil, Iran, Iraq and Syria. Meanwhile 350 million people are exposed to the parasite. The number of new cases of CL has reached to 1.5 million people in the word (*World Health Organization Control of leishmaniases* 2010). In Iran, CL distributes in some geographical locations such as north-east (Hajjaran *et al* 2004), center (Sharifi *et al* 2011), west, east and south (Farahmand *et al* 2011, Fazaeli *et al* 2009). Two species of *Leishmania* are involved in CL infections in Iran. *L. major* is causative agent of zoonotic cutaneous leishmaniasis (ZCL) and *L. tropica* causes anthroponotic CL (ACL). The classic diagnostic techniques for cutaneous leishmaniasis have a number of limitations. Microscopic examination of

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skin scrapings has limited sensitivity, particularly in chronic lesions. In vitro culture techniques are susceptible to microbiologic contamination and since certain strains grow better than others in vitro, the dominant strains can be inadvertently selected when culturing mixed infections (Navin *et al* 1990). The Montenegro skin test detects specific cutaneous delayed-type hypersensitivity but cannot distinguish between current and past infection. Serologic diagnostic techniques present drawbacks that include the cross-reactivity of leishmanial antigens with antibodies induced by other kinetoplastids (Armijos *et al* 1990) as well as poor sensitivity due to the low antibody titer characteristic of cutaneous leishmaniasis. Species identification has been conventionally achieved using isoenzyme electrophoresis (zymodeme analysis) or monoclonal antibodies (serodeme analysis) (Kreutzer *et al* 1980). Zymodeme analysis is a lengthy and expensive process that requires large-scale cultivation of parasites. Monoclonal antibodies are useful for identification of species in cultured strains but are not as amenable to direct analysis of clinical specimens. Recently, conventional parasitological and serological techniques have been integrated by the more sensitive and specific polymerase chain reaction (PCR) assay (Grimaldi *et al* 1987, Leontides *et al* 2002, Manna *et al* 2008, Strauss-Ayali *et al* 2004, Schonian *et al* 2003). PCR has been shown to overcome problems such as the low sensitivity found with microscopic examination of tissue smears, and the limited predictive value of serology where the results may be affected by persistent antibodies or immunosuppression (Leontides *et al* 2002). During the past two decades epidemiological aspects of cutaneous leishmaniasis have been increasingly changed in Iran. These changes have been motivated through the population movement between the urban and rural areas, the migration of the neighboring countries including Afghanistan and Iraq. The rural cutaneous leishmaniasis is one of the most important health challenges in Iran as in most of the rural regions; the disease is prevalent in 17 provinces of 30 provinces in Iran. Isfahan and TurkmanSahra were the most important centers of cutaneous leishmaniasis of the villagers in Iran (Rassi *et al* 2008, Yaghoobi-Ershadi 1997).

This study has been conducted with the purpose of identifying dominant *Leishmania* parasite species in Isfahan using the PCR method because any effective control programmes should be based on the accurate baseline information about pathogen species.

MATERIALS AND METHODS

Sample collection. This study was conducted from September 2010 to September 2011. All procedures were performed under consent of patients. Sampling was accomplished by scraping doubtful cutaneous lesion of 124 patients. The research was done on the population referring to the Sidiqay Taheray center for skin disease and Leishmaniasis and population in this area with suspicious lesion of Leishmaniasis with positive direct smear from lesion was found. Patients with suspicious lesion of Leishmaniasis and positive direct smear from lesion were selected.

DNA extraction and PCR. Samples were cultured in NNN and RPMI 1640 media (John et al 2006). Total DNA was extracted from RPMI 1640 cultured samples and promastigote of *Leishmania*. To extract DNA, Phenol-chloroform method was applied. 500µl lysis buffer was added to parasite sediment (Surcrose 0.32 M, SDS 1%, Tris-Hcl 10mM, Mgcl2 5mM) and it was placed in water bath at 37°C .Then, an equal volume of the solution, 500µl phenol was added and it was centrifuged for 5min at 8000rpm. After adding 500µl chloroform to the supernatant and centrifuging at 8000 rpm, the supernatant was removed 0.1 of the liquid, 3 M sodium and two volumes of 100% ethanol were added and it was kept in freezer -20 °C. Then, it was centrifuged for 10 min at 12000 rpm and 100µl alcohol 80% was added and it was centrifuged for 2 min at 12000 rpm. After removing the supernatant, the sample was added for 5-10 min in incubator and then 30µl sterile deionized distilled water was added. Negative and positive control and clinical samples was applied

for PCR in the same condition. Positive control was prepared from Isfahan skin diseases and leishmaniasis research center. In the next step, standard PCR was carried out using classic protocol. Primer pair (Primer1: 5'_ TCGCAGAACGCCCCTACC_3' and Prime2: 5'_ AGGGGTTGGTGTAAAATAGGC_3') was used that generate products with size of 620 bp for *L.Major* and 830bp for *L. tropica* and was specific for conserved sequences of kDNA of Leishmania (Mahboudi *et al* 2001). For amplification, 2.5 µl of DNA sample was added to 22.5 µl of reaction mixture containing KCl 50 mM, Tris buffer 10 mM (pH 8.3), 0.2 mM each deoxynucleotide triphosphate, MgCl2 1.5 mM, DMSO 10.5%, tetramethyl ammonium chloride 50 mM, betaine 0.6 M, dithiothreitol 1mM, LU-5A probe 0.4 µM, 0.2 µM each 3' primer (LM-3A, LB-3C and LC-3L) and, Taq DNA polymerase 0.04 U/µl (Sigma). Amplification was incubated in a Thermolyne-Amplitron II thermal cycler, using an initial denaturation step of 95 °C x 5 min, followed by 35 cycles of 95°C x 30 s, 54 °C x 45 s, and 72°C x 30 s with a final extension at 72 °C x 5 min. Finally, 8 µl of the amplification products were analyzed by electrophoresis on 1.5% agarose gel in TBE buffer (89 mM Tris borate, 2 mM EDTA, pH 8.3) containing ethidium bromide 0.5 µg/ml. The amplification products were visualized under UV light and the gels documented by EPS-600Z cameras.

RESULTS AND DISCUSSION

From 124 patients, 111 (89.51%) cases were infected as *L. major* and 12 (9.67%) cases were infected by *L. tropica*, However only in one patient simultaneous infectious with both *L. major* and *L. tropica* was identified. *L. major* was the most prevalent species in the studied patients (p-value<0.001). Epidemiological results related to the information obtain from the questionnaire and determined species showed that: from 124 patient from whom the samples were taken, 10 of them were less than 10 years old. 48 patients were in the age group of 10-19. 32 patients were between 20-29 years old.13 of them was between 30-39 and 7 patient was 40-49 years old and 14 patient was >50 years old among these ages. One month old was the least and maximum age was 65 and the mean age was 29 and the maximum pottion was in 10-19 years old group (Table 1).

Table 1. The frequency of *L. tropica* and *L. major* based on different ages.

Infection with *L.tropica* and *L.major*	Infection with *L. tropica*	Infection with *L. major*	Number of patients	Age of patients
number	number	number		
0	0	10	10	10>
0	2	46	48	10-19
1	6	25	32	20-29
0	1	12	13	30-39
0	1	6	7	40-49
0	2	12	14	50>

From 124 patient , 75 were male and 49 female, from 75 male patient 67 were infect with *L. major* and 8 of them infected with *L. tropica* and from 49 female patient 45 had *L. major* and 4 had *L. tropica* (Table 2). Out of 124 patients, 66 had lesion on hand, 32 in the leg, 18 patients had lesion in the face and neck and 8 patients had lesion in trunk (Table 3). Leishmaniasis is caused by parasitic protozoa of the genus *Leishmania* and it is essential to identify the species of the parasite in epidemiological and clinical studies. All the species are similar physically (even by electron microscope) except a little difference in the size of the species. According to the considerable studies performed, it was defined that various species (despite the physical similarity), they are different in terms of molecular, biochemical and antigenic characteristics. Advance techniques as PCR are

proposed to identify various species. PCR is more precise compared to the microscopic observation of blood extension and other methods in identification of *Leishmania* species.

Table 2. The frequency of *L. tropica* and *L. major* among males and females.

Infection with *L. tropica* and *L. major*	Infection with *L.tropica*	Infection with *L.major*	Number of patients	sex
number	number	number		
0	8	67	75	**male**
1	4	44	49	**female**

PCR method is precise in discriminating pathogenic and non-pathogenic species. Brustoloni et al. 2007 evaluated the sensitivity and specificity of PCR method compared to other experimental methods. PCR showed the highest sensitivity (92.3%) and had good specificity (97.5%) (Brustoloni *et al* 2007).

Table 3. The frequency of *L.tropica* and *L.major* based on different part of body.

Infection with *L. tropica* and *L. major*	Infection with *L. ropica*	Infection with *L. major*	Number of patients	Lesion location
number	number	number		
1	5	60	66	hand
0	3	29	32	leg
0	0	8	8	trunk
0	4	14	18	Face and neck

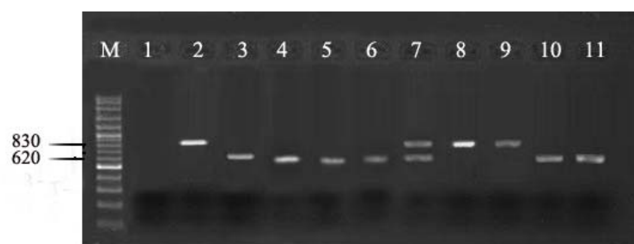

Figure 1. Polymerase chain reaction (PCR) profiles obtained from the *Leishmania* stocks: Lane1 negative control; lane 2 positive control for *L. tropica*; lane 3 positive control for *L.major*; Lane 4,5,6,10,11 are *L.major*; lane 8, 9 are *L.tropica*; lane7 is infection with both *L. tropica and L. major.*

As it was shown in frequency distribution of the patients in age group, 10 of the subjects were less than 10 years old. 48 patients were in the age group of 10-19. 32 patients were between 20-29 years old.13 of them was between 30-39 and 7 patients were 40-49 years old and 14 patients were>50 years old among these ages. From 124 patients, 75 were male and 49 female. Out of 124 patients, 66 people had lesion on hand, 32 in the leg, 18 patients had lesion in the face and neck and 8 patients had lesion in trunk. The major injury was observed in the hands and the result was consistent with the results of the study performed by Shiie et al. 2012. By the investigation of 137 infected subjects showed that there were 74 (54%) men and 63 (46%) women. The results of the study showed that the mean lesion was 2 and the infection duration was 97 days and 62.8 % of the cases were dedicated to lesion on hand (Shiee et al 2012). Azizi et al. 2012 by microscopic and Nested-PCR detected the smear species of cutaneous leishmaniasis. They showed that the only smear species in southern province of Iran was *L.major* (Azizi et al 2012). Based on the results of amastigot species by PCR, from 124 patients, 111 (89.51%) cases were infected as *L.major* and 12 (9.67%) cases were infected by *L.tropica*, However only one patient (0.8%) was infected by both *L.major* and *L.tropica*. The results of the present study showed that *L.major* was the most prevalent species and it showed that *L.tropica* is local in Isfahan region. It can be said that population changes, migration and climatic changes caused that clinical forms of the disease were different. Mesgarian et al. 2010, applied PCR method to detect *Leishmania* species

in Gonbad Kavus. The results of the study showed that the tissues of the patients with cutaneous leishmaniasis were *L.major* (Mesgarian *et al* 2010). Rahbarian et al. 2009 in Gonbad-e Qabus, among 6990 inhabitants of 5 villages, 62.9% were identified as scars and 1.5% as active lesions. Individuals 11 to 20 years were the most highly infected age group. All the parasite isolates were *Leishmania major*. (Rahbarian *et al* 2009). In a study performed in Mashhad in 2010, of total 21 samples, 19 cases were *L.tropica* and 2 cases were *L.major*. The study showed that besides *L.tropica* species, *L.major* species is observed but *L.tropica* was prevalent (Mahmoodi *et al* 2010). The determination of the species of *Leishmania* in epidemiological studies to determine the main vector, definite source and human infection and their relationship with each other, the evaluation of different vaccines provided against leishmaniasis, the determination of treatment effect of various methods and the selection of a good strategy to control and prevent leishmaniasis are necessary.

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